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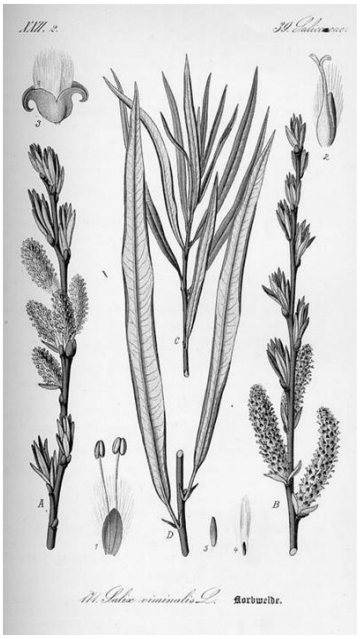

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Abstract

Background and aims *Salix viminalis,* which is a C3 perennial crop, is one of the promising candidates for bioenergy production. It is easy to propagate by stem cutting and is able to re-sprout after each harvest. In addition, *S. viminalis* can produce high biomass yield within a short time frame. The broad genetic base of *Salix* is an advantage for the development of molecular markers for breeding purposes. Although *S. viminalis* has high water use efficiency, it has relatively high transpiration rate. Hence, it would be ideal if the crop is drought tolerant and able to produce optimal yield despite limited water availability. In order to understand the drought response of *S. viminalis* between well-watered and drought conditions, we have studied the phenotypic variation of *S. viminalis* genotypes in well-watered and water stress conditions.

Materials and methods A complete randomized block design was used for the greenhouse experiment with three blocks as the control blocks and another three drought-treated blocks. In total, 296 genotypes of *S. viminalis* and were used for the study. The phenotypic data which included height growth during treatment, crown length, stem dry weight, leaf dry weight and the number of sylleptic shoots was analyzed using ANOVA by JMP software. Two indexes were constructed namely, Drought Response Index (DRI) and Stem Biomass Allocation Index (SBAI). Three statistical models were used to analyze Genotype x Treatment (GxT) interaction effect, heritability and genetic variation. Also, the relationship between the phenotypic traits was examined by Pearson pairwise correlation.

Results There was significant difference between the treatments in all the measured traits. Also, there was significant difference among *S. viminalis* genotypes in response to water stress condition. Though the total biomass was reduced, the biomass allocation to stem seemed to be less influenced by water stress. Height growth during treatment, crown length and dry weights had higher GxT interaction effect among the genotypes. In contrast, the GxT interaction effect was not significant in stem biomass allocation. The number of sylleptic shoots had stable heritability values ($H^2_{Genotype}$) across the treatments while stem biomass allocation had the highest heritability in both treatments. In addition, there was a decrease in the genetic variation within the population for all the traits in drought condition, except for the number of sylleptic shoots which experienced an increase of genetic variation.

Conclusion We suggest that the number of sylleptic shoots should receive more attention in *Salix* breeding because it is positively correlated with the growth traits. Also, stem biomass allocation can be considered in breeding for higher biomass production. It will be challenging if height growth, crown length and dry weight were considered in breeding, because it is harder to estimate the yield since these traits have high GxT interaction effect within the population.

Keywords: *Salix viminalis*, well-watered, drought, Genotype x Environment interaction (GxE), Broad sense heritability (H^2), Phenotypic correlation

Sammanfattning

Bakgrund och syfte *Salix viminalis* är en flerårig C3 växt och en lovande kandidat för produktion av bioenergi. Arten är lätt att föröka vegetativt med förvedade sticklingar och den skjuter nya stubbskott efter varje skörd. Dessutom är *S. viminalis* snabbväxande och har en hög biomassaproduktion. Inom *Salix* finns en hög genetisk variation vilket är en fördel vid utveckling av molekylära markörer inom växtförädlingen. Trots att *S. viminalis* har en hög effektivitet i vattenutnyttjande, har den en relativ hög transpiration. Det skulle därför vara idealiskt att ta fram Salixsorter som tål torka och samtidigt har en hög biomassaproduktion trots en begränsad tillgång på vatten. För att studera torkstressresponsen hos *S. viminalis*, har vi studerat den fenotypiska variationen hos *S. viminalis*-genotyper i två olika behandlingar; god vattentillgång och vattenstress.

Material och metoder En fullständigt randomiserad blockdesign användes i ett växthusförsök med tre kontrollblock, d.v.s. god vattentillgång, samt tre torkbehandlade block. Totalt användes 296 genotyper av *S. viminalis* för studien, varje genotyp var representerad en gång i varje block. Egenskaper som mättes var höjdtillväxt under behandling, kronlängd, stammens torrvikt, bladens torrvikt och antalet sylleptiska skott. Alla data analyserades med hjälp av ANOVA i programmet JMP. Två index konstruerades, dels ett tork-respons-index, dels ett index för stam-biomassa-allokering. Tre statistiska modeller har använts för att analysera interaktionen mellan genotyp och behandling (GxB), heritabiliteter i vid bemärkelse och genetisk variation. Dessutom studerades fenotypiska korrelationer mellan egenskaper.

Resultat Alla egenskaper skilde sig signifikant mellan behandlingar. Dessutom var det en signifikant skillnad i torkstressrespons mellan *S. viminalis*-genotyper. Den totala biomassan minskade under torkstress men biomassaallokeringen mellan stam och blad förändrades inte mellan behandlingarna. Interaktionseffekten mellan genotyp och behandling (GxB) var signifikant och hög för egenskaperna höjdtillväxt under behandlingen, kronlängd och torrvikter. Däremot var GxB effekten inte signifikant för biomassaallokering. Allokering av stambiomassa hade den högsta heritabiliteten i båda behandlingarna medan antalet sylleptiska skott hade liknande heritabiliteter i de olika behandlingarna. Den genetiska variationen inom populationen minskade hos alla egenskaper i torkbehandlingen, med undantag för antalet sylleptiska skott där genetiska variationen ökade.

Slutsatser Vi föreslår att antalet sylleptiska skott bör uppmärksammas mer inom växtförädlingen av *Salix* eftersom den egenskapen är positivt korrelerad med tillväxtegenskaper. Även biomassaallokering kan användas inom växtförädlingen för ökad produktion av biomassa. Den höga interaktionseffekten mellan genotyp och behandling (GxB) för egenskaperna höjdtillväxt, kronlängd och torrvikt innebär att ett direkt urval för dessa egenskaper bör göras separat för torka respektive god vattentillgång.

Nyckelord: *Salix viminalis*, god vattentillgång, torkstress, genotyp x miljö interaktion, heritabilitet i vid bemärkelse (H^2), fenotypisk korrelation

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1. Introduction

Bioenergy is a form of renewable energy that derives from biological sources, and it has become popular in today's research (Karp and Shield 2008). Energy derived from crops is a promising prospect as this can add value to some neglected crops. The crops that can be used for bioenergy production is called bioenergy crops. Currently, ongoing research in bioenergy crops is aiming for a slow replacement of energy that derives from coal, crude oil or nuclear power (Oliver *et al*. 2009). In future, the demand for fuel will increase due to the fast-growing world population and this could be one of the major challenges in the human society. In addition, it is predicted that world climate might become warmer and drier and this may affect biomass production of the crops, especially in the northern latitude region (Oliver *et al*. 2009). Even though there might be an elevation of carbon dioxide (CO_2) concentration that could enhance photosynthesis efficiency, drought might mask the effect of the elevated CO_2 and has greater impact on plant growth (Chaves *et al*. 2003). This could greatly affect plant yield that is important for food security and bioenergy production. So, in order to cope with future climate and world's energy security, bioenergy crops with drought tolerance and high biomass yield are most desirable.

In the past few decades, most of the bioenergy are derived from wood and agricultural crops such as maize, wheat, sugar cane and sugar beets. They are the 1st generation bioenergy crops. However, due to the growing world population, most of the agricultural crops are diverted to food production. So, during the last few years, 2nd generation bioenergy crops have become the main target for bioenergy production. Among the 2nd generation bioenergy crops, *Salix* which is a perennial C3 species, has a huge prospective. The common name for *Salix* is willow and it is often cultivated in short-rotation system (Rönnberg-Wästljung *et al.* 1994). With over 300 species, *Salix* is prevalent in the northern and southern hemispheres, except in Australasia and New Guinea (Åhman and Larsson 1994; Berlin *et al.* 2011). Since 1970s, *Salix* received attention for bioenergy production due to its natural qualities (Rönnberg-Wästljung *et al.* 1994). One of the qualities is that it has a relatively broad genetic diversity within the genus, which could

provide many options for the selection of desirable genotypes in breeding. In addition, the broad genetic variation is an advantage in genomic research for developing molecular breeding tools such as molecular markers (Åhman and Larsson 1994; Kopp *et al.* 2002). Linkage maps and quantitative traits loci (QTL) analyses of different *Salix* species are available and this could be used in molecular breeding (Tsarouhas *et al*. 2002; Rönnberg-Wästljung *et al.* 2003; Rönnberg-Wästljung *et al.* 2005). Moreover, it was suggested by Berndes (2007) that *Salix* could be part of the multifunctional environment system that is beneficial to the ecosystem. For example, *Salix* can be served as a vegetation filter for water purification and prevent nitrogen leakage from the soil. Among all the *Salix* species, *Salix viminalis* is one of the main species and it has a wide natural distribution ranging from west of Ireland and United Kingdom to the east of Siberia (Berlin *et al*. 2011). *S. viminalis* is considered as a bioenergy crop because it produces high biomass within short period and is able to re-sprout after each harvest (Sennerby-Forsse *et al*. 1992; Gullberg 1993; Rönnberg-Wästljung *et al.* 1994). Also, it is vegetatively propagated by stem-cuttings and this makes the planting process easier (Rönnberg-Wästljung 2001). Thus, with all these good qualities, *S. viminalis* is considered as a promising candidate for bioenergy production.

In order to become the ideal bioenergy crop, it should have the ability to produce high biomass within a short time frame, be resistant to pests and diseases, be drought tolerance and ecosystem-friendly (Karp and Shield 2008). According to Lindroth and Båth (1999), despite the cool and humid Scandinavian climate, water availability is the limiting factor for biomass production in *Salix*. This is because *Salix* has high transpiration rate and is inefficient to maintain xylem integrity during water stress condition (Pockman and Sperry 2000). When water availability is limited, it reduces root-to-soil contact and hence, decreases water uptake. Also, this increase the risk of air entering the xylem and causes xylem cavitation that finally leads to embolism - air filling (Wikberg 2006). Thus, xylem integrity is affected during water stress condition. So, in order to adapt towards warmer and dryer climate in future, drought tolerance is one of the important traits that need to be considered in *S. viminalis* breeding. A drought

tolerance crop can survive with low tissue water potential and is still able to produce high biomass production under water stress condition.

From the agricultural viewpoint, drought or water stress is a form of abiotic stress which is described as insufficient water availability that could restrict optimum genetic expression of the crops (Mitra 2001). In water stress condition, the physiological processes are affected and this is reflected in its phenotype. In general, water shortage causes reduction in cell size, reduced water use efficiency and a reduction in biomass production (Acquaah 2007). Drought response of *Salix* can be displayed in several quantitative traits, such as height and dry weights. Also, in drought condition, some morphological changes such as reduced leaf size area, increased root depth and root length distribution, decreased shoot to root ratio in *Salix* seedlings were observed in a greenhouse experiment conducted by Van Splunder *et al*. (1996).

In *Salix*, most of the biomass is derived from stem. However, during water stress condition, growth source is mainly channeled to roots rather than to leaves (Weih *et al.* 2011; Wikberg and Ögren 2007). This re-allocation increases root growth and promote wider and deeper root distribution. Even though this re-allocation will increase drought tolerance, it reduces the allocation of resources to stem and leaf. Consequently, this decreases the harvestable biomass. Also, the allocation between stem and leaf is affected during water stress condition. When water is limiting, this encourages the closure of stomata to prevent further water loss through transpiration (Wikberg and Ögren 2004). However, the closure also reduces photosynthetic efficiency and less carbon is assimilated to leaf. Leaf is the main photosynthesis site that generates growth resources for the whole plant. If the photosynthetic efficiency decreases, this affects the allocation pattern to the other parts of the plant. Thus, during drought, the allocation to stem is reduced and this affected stem growth.

Sylleptic shoot is another unique trait that is important in the life-cycle of *Salix*. Sylleptic shoot is the newly developed lateral axis without the apical meristem passing through a dormant period (Remphrey and Powell 1985). *Populus* and *Salix* belong to the same taxonomic family. The growth characters and phenotypic traits of *Populus* are extensively studied and *Populus* is also one of candidates for bioenergy production (Karp and Shield 2008). One of the traits that are well-studied in *Populus* is the sylleptic shoots. Based on Marron *et al.* (2006), sylleptic branching in poplar is the major factor that contributes to high leaf area index. Sylleptic shoots play an important role in canopy architecture and is one of the determinants for dense plantation in *Populus* (Dillen *et al*. 2009). However, it was suggested that sylleptic shoots in *Populus* and *Salix* served different functions. Sylleptic shoots in *Salix* are relatively smaller and often drop off at the end of growing season (Verwijst and Wen 1996; Rönnberg-Wästljung and Gullberg 1999). In contrast, sylleptic shoots in *Populus* are larger and permanent (Rönnberg-Wästljung and Gullberg 1999). As compared with the other *Salix* species, *S. viminalis* has a higher number of sylleptic shoots. According to Wikberg and Ögren (2004), sylleptic shoots are more drought sensitive than the main stem. So, during water stress condition, sylleptic shoots will shed off or wilt. This could improve water use efficiency and also to reserve more growth resources to the root. Thus, sylleptic shoots are considered as the burden during drought.

Genotype and environment (GxE) interaction is one of the crucial aspects in plant breeding. If a genotype undergoes change of ranking from one environment to another, this indicates that there is an interaction between the genotype and the environment (Acquaah 2007). In **Fig. 1a**, there is no GxE interaction as Genotype A is consistently out-performed Genotype B. In **Fig. 1b**, there is GXE interaction as Genotype C and D perform differently in the two environments. Genotype C is more productive in environment *Y* while Genotype D is more productive in environment *X*. In this case, there is a change in the genotype ranking from one environment to another.

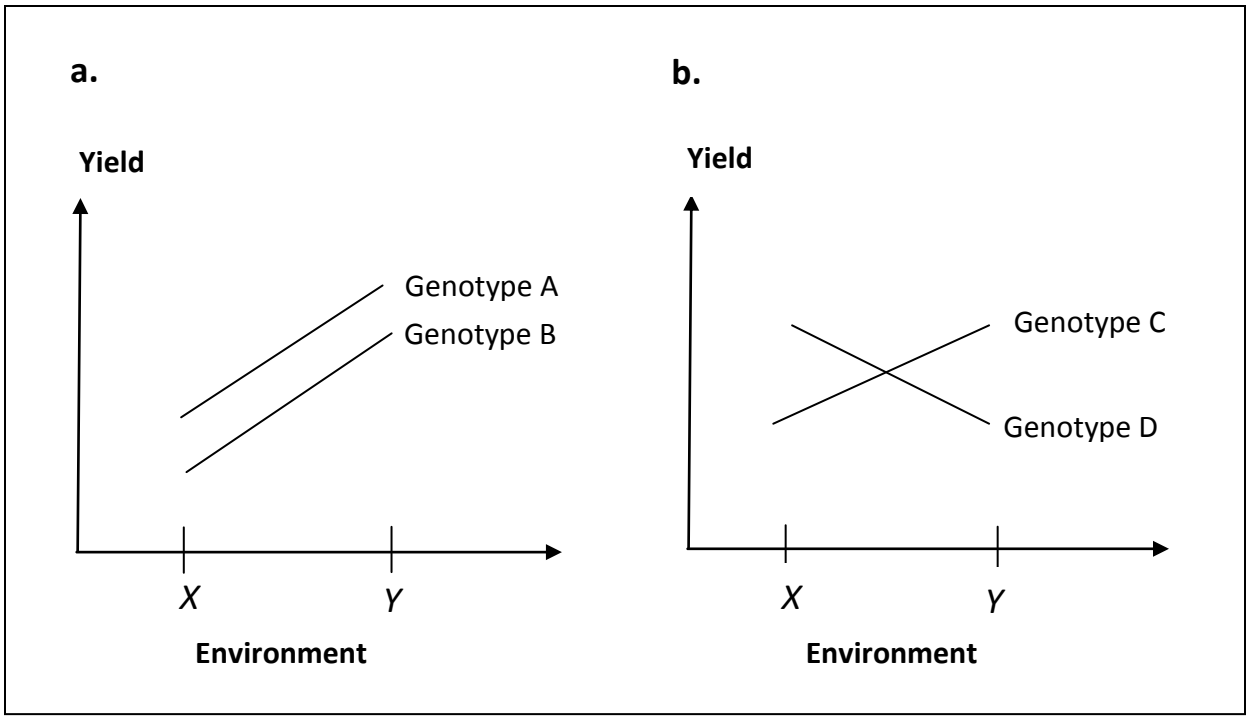


Fig. 1: GxE interaction illustration. **a)** There is no GxE interaction since there is no changing of the genotype ranking as Genotype A has consistent better yield than Genotype B across the environments. **b)** There is GxE interaction as there is changing of genotype ranking (i.e. Genotype C has better yield while Genotype D is less productive in environment *Y*).

In crop breeding, it would be ideal to select the genotype that has consistent high yield in different environments. Also, if there is GxE interaction effect in the population, zone breeding could be considered, in which the field is divided into different areas for different genotypes. This could optimize the yield of each genotype.

Broad sense heritability (H^2) is one of the key components in breeding. It gives a measurement of the genetic contribution to the phenotypic variation within a population in a particular environment (Acquaah 2007). If a trait has high heritability, this means that the phenotypic variation of the trait is mostly due to the genetic difference among the individuals in the population. If a trait has low H^2, this shows that the trait is more influenced by environmental

factors rather than genotypic factors. In this case, the trait will have a slow response towards selection and it will have low genetic gain in breeding.

The relationship between the phenotypic traits is one of the important elements that have to be considered in breeding. The observed phenotypic trait is the result of the genotypic expression in an environment. Thus, it is the combination of genetic and environmental factors that contributes to the phenotypic correlation (Acquaah 2007). Phenotypic correlation varies dependent on heritability. Genetic factor become the main part of the correlation when there is a high heritability. In contrast, with low heritability, environmental factor becomes the major aspect in the phenotypic correlation. In breeding, it is important to consider the correlation between traits as this can provide information on how to select the desired traits. For example, if two traits are genetically correlated, selection of one trait influences also the correlated trait.

The existing quality of a crop can be improved through breeding. Preliminary selection of the potential genotypes is one of the primary steps in breeding. Normally, this can be done in nursery with relatively low cost and subsequently, field trails are carried out to evaluate the performance of the selected genotypes (Sennerby-Forsse *et al*. 1992). Breeding programs of *Salix* have been established in many countries. Sweden, United Kingdom and United States are the leading countries that have the most extensive *Salix* breeding programs. They all have the common vision – to improve the breeding materials of *Salix* for high yield, resistant to diseases and pests, and the ability to tolerate different abiotic and biotic stresses. All these attributes are intended to promote *Salix* as one of the bioenergy crops in future.

For *S. viminalis* to become an ideal bioenergy crop, genotypes that are able to withstand water stress and still able to produce high biomass are needed. Thus, the aim of this project is to study the phenotypic variation of *S. viminalis* in well-watered condition and drought condition

by examining several phenotypic traits such as height growth during treatment, crown length, stem dry weight, leaf dry weight, total dry weight and the number of sylleptic shoots. By understanding phenotypic responses between well-watered and drought conditions, it will be a foundation for drought tolerance breeding in *S. viminalis*. To fulfill the goal of this project, we would like to address the following questions:

i. Is there a **Genotype x Treatment (GxT) interaction** and do the genotypes **respond** differently across the treatments?
ii. Are there any genotypes with **better performance (i.e. higher yield)** under drought condition compared to the well-watered condition?
iii. What is the **broad sense heritability (H^2)** for the phenotypic traits in each treatment?
iv. What is the **relationship** between the phenotypic traits and how can this be used in breeding?

We believe that the results from this project could be the first step to select desirable genotypes for *Salix* breeding programs. Furthermore, the outcome of this study will be used in another study to identify the candidate molecular markers for drought tolerance breeding in *Salix*.

2. Materials

In spring 2009, 385 *S. viminalis* stem cuttings originated from different European countries (i.e. Belgium, Czech Republic, Finland, Germany, Sweden, Poland and United Kingdom (**Fig. 2**) were planted in an experimental field at Pustnäs, Uppsala (Sweden). Microsatellite and the SNP markers were used to analyze the genetic profile of each genotype thus revealing that some of the *S. viminalis* genotypes shared identical genetic profile. A total of 296 genotypes of *S. viminalis* were used for the phenotypic analysis.

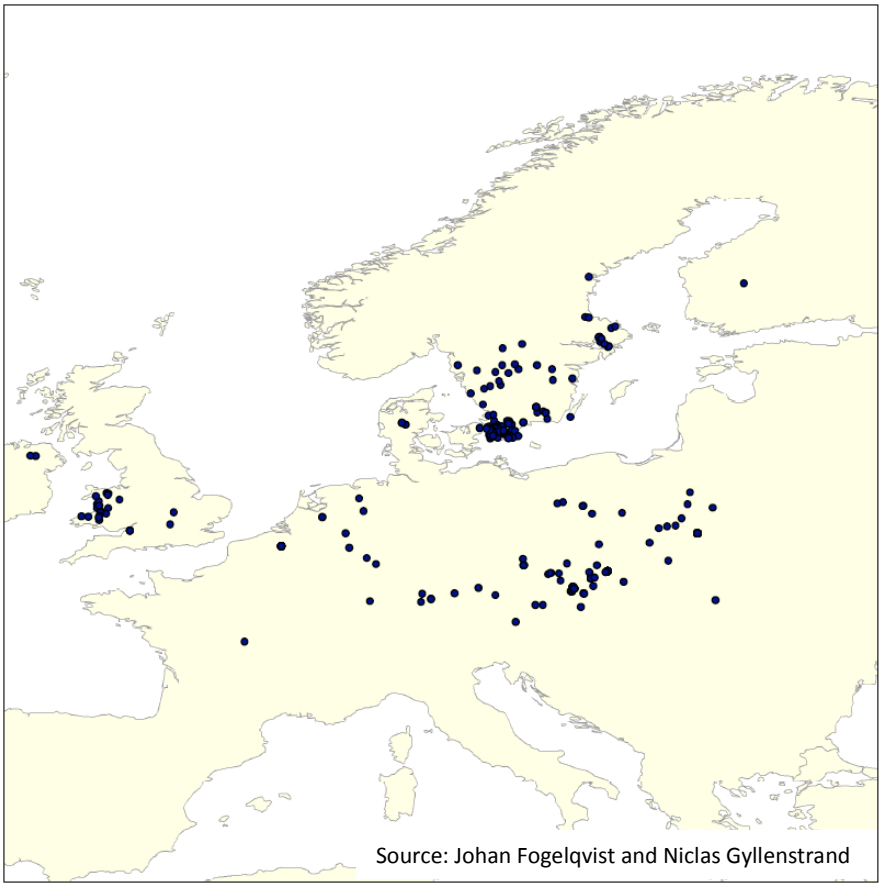


Fig. 2: The country of origin of each *S. viminalis* genotype – materials for the greenhouse experiment

3. Methodology

3.1 ***Greenhouse experimental design***

One shoot of each genotype was harvested in February 2011 and six stem cuttings (approximately five cm) of each genotype were prepared for a greenhouse experiment. In late February 2011 the greenhouse experiment was initiated. The experiment was a complete randomized block design **(Fig. 3**) where all genotypes were planted once in each block. Three litre pots filled with Weibulls 'Kron Mull' (organic matter 95%; pH 5.5-6.5; 180 g/m3 N, 110 P, 195 K, 260 Mg, 100 S, 2000 Ca) were used in the experiment. Three blocks were well-watered (i.e. control) while another three blocks were drought-treated. Six stem cuttings from each genotype were randomly planted in pots with one cutting per block. In total there were 2136 plants in the experiment. The average temperature of this experiment was around 20 °C.

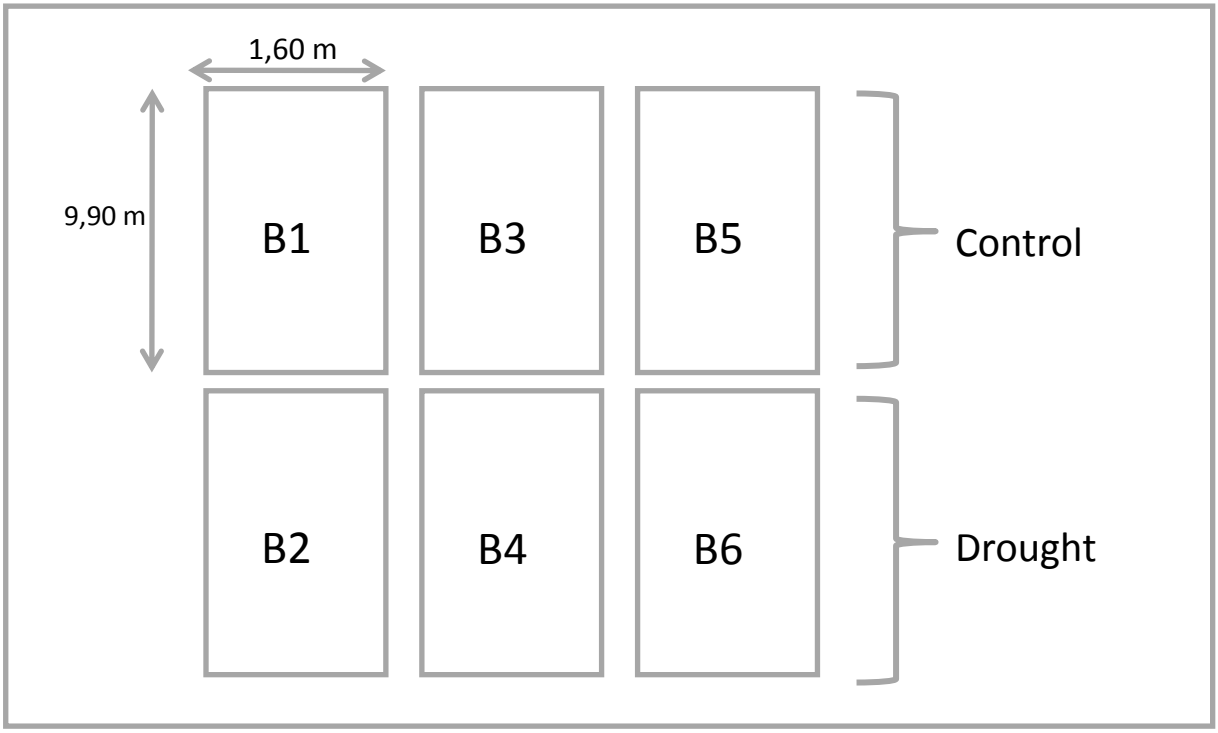


Fig. 3: Greenhouse experiment layout – a complete randomized block design with Block 1 (B1), Block 3 (B3) and Block 5 (B5) as the control block; while Block 2 (B2), Block 4 (B4) and Block 6 (B6) were the drought-treated blocks.

At the start of the experiment, all the six blocks received equal amount of water and were watered once a day to field capacity. After nearly five weeks, drought treatment was applied to three of the blocks. The duration of the drought treatment was seven weeks. The treated blocks were watered every day with an amount of water that was just sufficient for the plants to survive and grow. To estimate the average difference in the amount of water given to the two treatments, ten randomly selected pots from each block were weighted repeatedly during the treatment period. On average, the drought treated plants had 42% less water supply compared to the control plants. The control blocks were watered to field capacity throughout the experimental period.

3.2 ***Phenotypic data***

Prior to the drought treatment, initial height of each plant was measured. In mid-May 2011 (after seven weeks of the treatment), all the plants were harvested and several phenotypic traits were measured for each plant (i.e. plant height at harvest, height to crown base and number of sylleptic shoots). In addition, stems and leaves of each plant were separated and oven-dried at 70 ^{o}C for at least 24 hours. Dry weights of leaf and stem for each plant were measured. All the height measurements were in centimeter (cm), while the measurement unit for dry weight was in gram (g). In addition, during harvest, plant damages due to drought treatment were observed. After collecting the phenotypic data, Stem Biomass Allocation Index (SBAI) and Drought Response Index (DRI) were constructed for each genotype.

i) Stem Biomass Allocation Index (SBAI)

SBAI was constructed to examine the biomass allocation to stem of each genotype in both treatments. The stem dry weight and total above ground dry weight (total of stem and leaf dry weights) of each plant were used for the SBAI index calculation.

$$\text{SBAI} = \text{Stem dry weight} / \text{Total above-ground dry weight}$$

ii) Drought Response Index (DRI)

In order to assess the difference in performance of each *S. viminalis* genotype in well-watered compared to drought condition, a Drought Response Index (DRI) was constructed for each genotype in each trait. In each treatment, the mean phenotypic data was used for the index calculation.

$$\text{DRI} = \text{Mean phenotypic data in drought} / \text{Mean phenotypic data in control}$$

If DRI > 1, this means that the genotype performs relatively better in drought than in control. If DRI < 1, this indicates that the genotype performs better in control than in drought treatment. The genotype that has DRI = 1 means it has a stable performance across the different treatments.

3.3 ***Data analysis***

JMP ® version 9.0.0 was used to perform the statistical analysis. Prior to the data analysis, the pattern of distribution for the all phenotypic data was examined using Kolmogorov-Smirnov-Lilliefors (KSL) test.

Analyses of variance (ANOVA *F* test) were performed to examine the statistical significance for all the traits by using the following models. In consideration of the initial height difference among genotypes, height before treatment was included in the models as the covariate (C_i). The effects in the model would be considered as significance if $p < 0.05$.

Model 1 was used to investigate the statistical significance between the treatments, the difference among the genotypes and the GxT interaction.

Model 1

$$Y_{ijkl} = \mu + C_i + T_j + B_k[T_j] + G_l + (G \times T)_{lj} + \varepsilon_{ijkl}$$

where μ is the general mean, C_i is the covariate, T_j is treatment effect, $B_k[T_j]$ is the effect of block nested within treatment, G_l is the l genotype effect, $(G \times T)_{lj}$ is genotype by treatment interaction effect, and ε_{ijkl} is residual random error. All the effects in this model were considered as fixed.

To examine the strength of GxT interaction, GxT quotient was calculated (Lindgren 1984). The variance components were obtained from Model 1. All the effects in the model were considered as fixed except for genotype effect (G_l) and the genotype x treatment $(G \times T)_{lj}$ which were regarded as random.

$$\text{GxT quotient} = \sigma^2_{GxT} / \sigma^2_{G}$$

The analysis result from Model 2 was used to identify the genotypes that performed statistically significant difference between the treatments. For a true and reliable testing, the significance level should be adjusted according to the multiple testing principles.

Model 2

$$Y'_{ij} = \mu + C_i + T_j + \varepsilon_{ij}$$

where μ is the general mean, C_i is the covariate, , T_j is the treatment effect, and ε_{ij} is residual random error. All the effects were considered as fixed.

In Model 1 analysis, if the test showed a significant GxT interaction in the phenotypic traits, several genotypes were selected to display the GxT interaction in graphs. The graphs could clearly display the change of ranking of the selected genotypes in well-watered and drought conditions. The selection of the genotypes for the GxT interaction plot was based on two criteria. Firstly, based on the DRI distribution histogram, the genotypes at the extreme ends of the histogram (i.e. DRI > 1 and DRI < 1) and genotypes with DRI = 1 were selected. Secondly, among the selected genotypes, only genotypes that showed significance in Model 2 analysis were chosen for the GxT interaction plot.

Model 3 was constructed to obtain the variance components for the heritability (H^2) and the coefficient of variance (CV_G)

Model 3

$$Y''_{ijk} = \mu + C_i + B_j + G_k + \varepsilon_{ijk}$$

where μ is the general mean, C_i is the covariate, B_j is the j block effect which regarded as fixed, G_k is the k genotype effect which regarded as random, and ε_{ij} is residual random error.

In this project, $H^2_{Genotype}$ was used to estimate the broad sense heritability of each trait in both treatments. All variance components were obtained from Model 3. The standard errors (SE) for the heritability values were obtained from the formula described by Singh *et al.* (1993).

$$H^2_{Genotype} = \sigma^2_G / [\sigma^2_G + (\sigma^2_\varepsilon / n_i)]$$

where σ^2_G is genetic variance component, σ^2_ε is residual variance component and n_i is mean number of individual per genotype (Nyquist 1991).

Coefficient of genetic variation (CV_G) was used to estimate the genetic variability of each trait in *S. viminalis* population exclusively for the environment. CV_G standardized the genotypic factors by diving them with the treatment mean of each trait (Marron *et al.* 2006). The standard deviation (σ_G) was obtained from Model 3.

$$CV_G\ (\%) = \sigma_G\ /\ \text{Treatment mean of each trait}$$

In both treatments, the relationships between the phenotypic traits were examined using Pearson's Pairwise Correlation. The strength of correlation was demonstrated in *r* value. The strength of correlation was defined as the following. If *r* is between 0.8 and 1.0, this means that it is a strong correlation. If *r* is between 0.5 and 0.7, the strength of correlation is considered moderate; while it is a weak correlation if *r* is less than 0.5. The significance of the correlation was determined by *p*-value. Mean value of each genotype was used for the analysis.

4. Results

The distribution pattern of all the phenotypic data was examined and in almost all cases, they were normally distributed. Both transformed (i.e. $\log_{10}$) and untransformed data were analyzed by JMP program. It was found that the analysis results of the transformed and untransformed data were similar. Hence, the original data (untransformed) was used for all the statistical analysis.

Table 1: Overview of all the phenotypic traits in both treatments – control and drought

Phenotypic traits	Control [1]				Drought [1]			
	Mean ± SE	SD	Min	Max	Mean ± SE	SD	Min	Max
Height growth during treatment (cm)	115.92 ± 1.14	36.59	5.00	209.00	96.48 ± 1.18	38.04	6.00	179.00
Crown length (cm)	120.09 ± 1.19	38.01	6.00	234.00	97.02 ± 1.07	33.74	4.00	177.00
Number of sylleptic shoots	5.01 ± 0.23	7.24	0.00	47.00	3.10 ± 0.17	5.41	0.00	39.00
Stem dry weight (g)	8.49 ± 0.19	6.00	0.07	31.34	5.61 ± 0.11	3.53	0.11	17.76
Leaf dry weight (g)	5.03 ± 0.09	2.87	0.02	14.82	3.87 ± 0.06	1.97	0.00	11.50
Total above ground dry weight [2] (g)	13.55 ± 0.28	8.75	0.09	45.49	9.53 ± 0.17	5.35	0.29	28.97
Stem biomass allocation (i.e. SBAI[3])	0.59 ± 0.003	0.09	0.23	0.78	0.58 ± 0.003	0.11	0.26	1.00

[1] **SE** – Standard error; **SD** – Standard deviation; **Min** – Minimum value; **Max** – Maximum value

[2] **Total above ground dry weight** – Total of stem and leaf dry weights

[3] **SBAI** – Stem biomass allocation index

In general, all the mean values of each phenotypic trait decreased in the drought treatment **(Table 1)**. However, the mean value of stem biomass allocation did not change much between the treatments. In addition, there were more plants with sylleptic shoots in control compared to drought treatment.

Table 2: ANOVA analysis based on Model 1. F-values were shown with the significance.

Sources	DF [1]	Height growth during treatment	Crown length	Number of sylleptic shoots	Stem dry weight	Leaf dry weight	Total above-ground dry weight [2]	Stem biomass allocation (i.e. SBAI [3])
Treatment	1	143.06 ***	211.72 ***	44.55 ***	264.48 ***	130.33 ***	214.01 ***	6.35*
Block[Treatment]	4	17.23 ***	24.38 ***	18.18 ***	32.67 ***	35.12 ***	34.18 ***	14.16***
Genotype	295	1.95 ***	1.82 ***	2.04 ***	1.85 ***	1.87 ***	1.79 ***	2.56***
Genotype x Treatment	295	1.56 ***	1.39 ***	1.36 ***	2.39 ***	2.05 ***	2.33 ***	1.10 (NS)
Covariate: Height before treatment	1	74.05 ***	73.54 ***	128.40 ***	471.06 ***	286.42 ***	422.40 ***	196.54***
Error	1413 -1461							

[1] **DF** – Degree of freedom

[2] **Total above ground dry weight** – Total of stem and leaf dry weights

[3] **SBAI** – Stem biomass allocation index

NOTE: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS – Non significance

The ANOVA analysis **(Table 2)** showed a significant difference between the treatments in all the phenotypic traits. Also, there were significant differences among the genotypes. Moreover, there was significance GxT interaction in all the phenotypic traits, except for stem biomass allocation.

Table 3: GxT Quotient for the different traits. Variance components were obtained from Model 1 with genotype effect (G_i) regarded as random.

Phenotypic traits	σ^2_G [1]	σ^2_{GxT} [2]	GxT Quotient ($\sigma^2_{GxT}/\sigma^2_G$)
Height growth during treatment	59.61	163.46	2.74
Crown length	61.86	108.30	1.75
Number of sylleptic shoots	2.98	3.26	1.09
Stem dry weight	0	5.14	-
Leaf dry weight	0	1.16	-
Total above-ground dry weight [2]	0	11.39	-
Stem biomass allocation (i.e. SBAI[3])	0.0013	0.0002	0.18

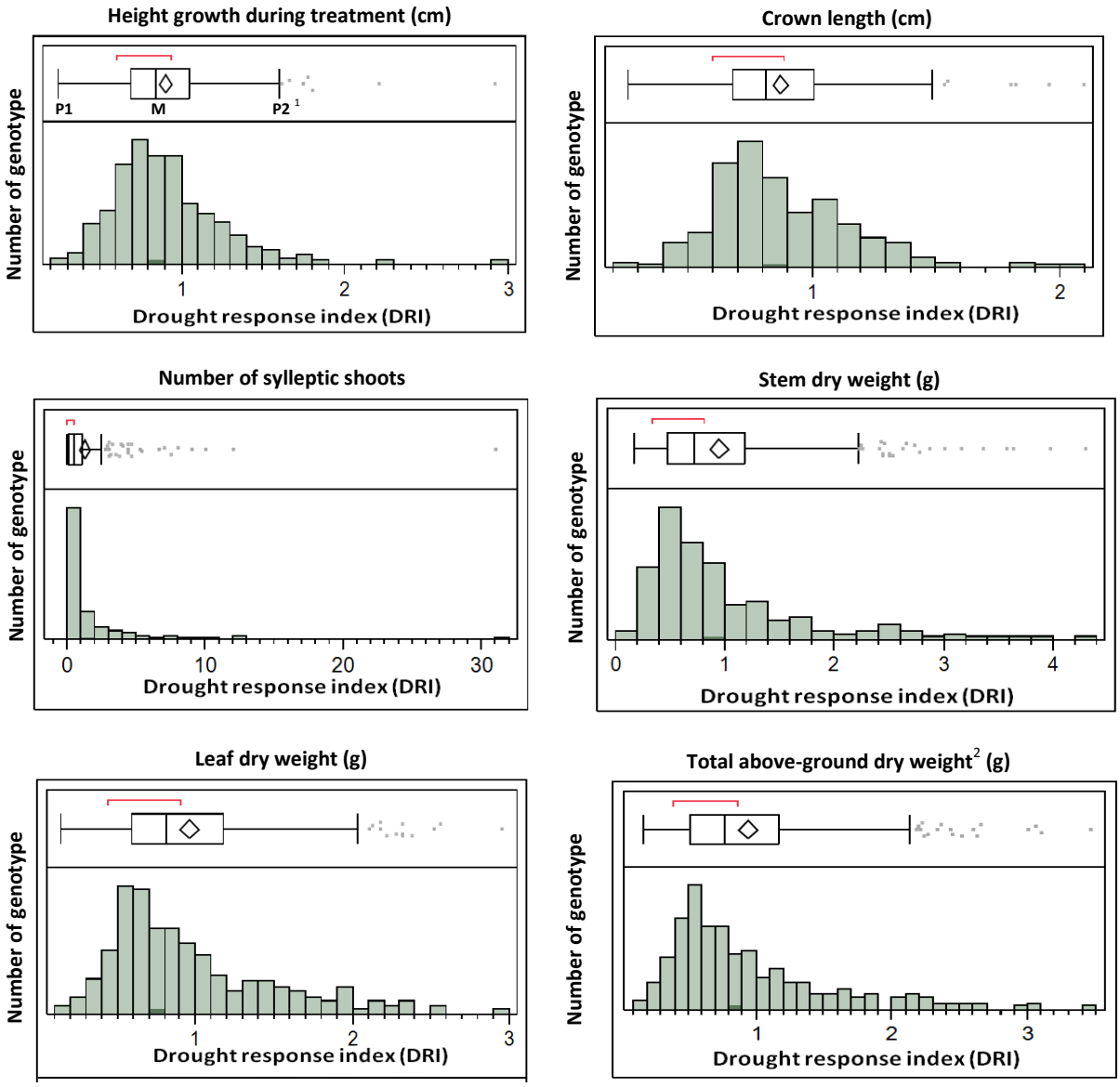
[1] σ^2_G – Genotypic variance; [2] σ^2_{GxT} – Genotype x Treatment interaction variance

[2] **Total above ground dry weight** – Total of stem and leaf dry weights

[3] **SBAI** – Stem biomass allocation index

NOTE: Negative variance component was considered as zero

The strength of GxT interaction was further tested by GxT quotient **(Table 3)**. Height growth during treatment had the highest quotient value (2.74); while stem biomass allocation had the lowest value (0.18). However, there was smaller genotypic variance (σ^2_G) in stem, leaf and total above-ground dry weights compared to their $\sigma^2_{GxT.}$ Also, stem biomass allocation (Stem / Total) had the lowest GxT quotient value. It is worth to mention that height growth during treatment and crown length had large difference between their genotypic variance (σ^2_G) and GxT variance (σ^2_{GxT}). In contrast, the variance components of the number of sylleptic shoots was not much different from each other ($\boldsymbol{\sigma^2_{GxT}}$ = 3.26, $\boldsymbol{\sigma^2_G}$ = 2.98).

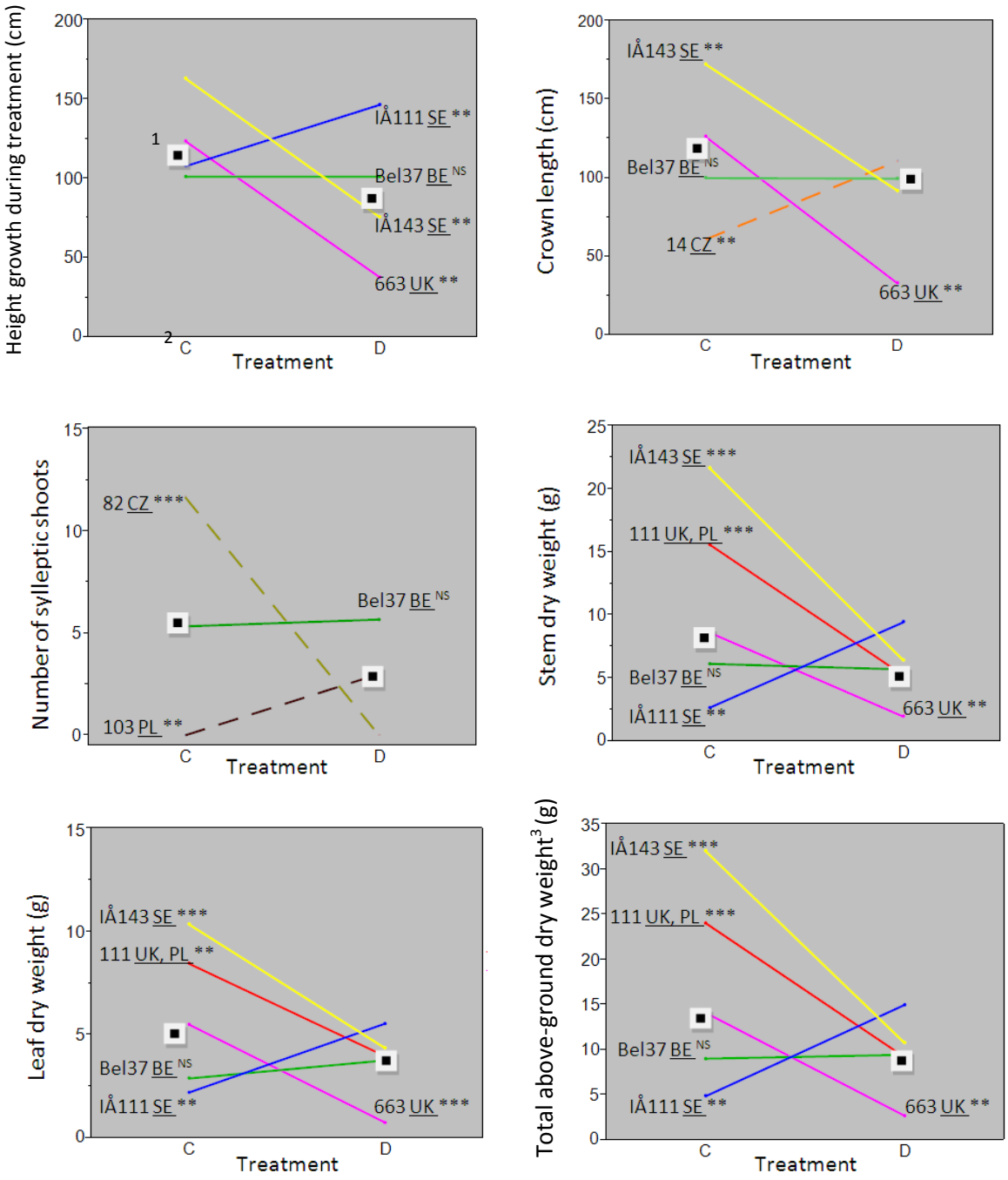


[1] **P1** – 25% percentile; **P2** – 95% percentile; **M** – Median

[2] **Total above-ground dry weight** – Total of stem and leaf dry weights

Fig. 4. Drought Response Index (DRI) distribution histograms for the phenotypic traits.

As showed in **Fig. 4**, Drought Response Index (DRI) distribution histogram were plotted to illustrate the response of the genotypes in well-watered and drought conditions. Majority of the genotypes had DRI < 1 and this indicated that most of the genotypes performed better in well-watered condition compared to drought condition.



1) ■ – Mean value of the treatment

2) **C** – Control (well-watered); **D** – Drought treatment

3) **Total above-ground dry weight** – Total of stem and leaf dry weights

NOTE: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS – Non significance

BE – Belgium; CZ – Czech Republic; PL – Poland; SE – Sweden; UK – United Kingdom;

Fig. 5 Genotype x Treatment (GxT) interaction plots of the selected genotypes for all the phenotypic traits across the treatments. Dotted line represents the genotype that appeared only once.

Several genotypes were selected to illustrate GxT interaction in all the traits except for stem biomass allocation, because it did not show significant GxT interaction in Model 1 analysis (**Table 2**). **Fig. 5** illustrated the GxT interaction among the selected genotypes in different traits. For example, in drought treatment, genotype 663 had lower performance in most of the traits. In contrast, it was interesting to discover that some genotypes performed better in drought treatment. For example, genotype IÅ111 had better performance in drought treatment compared to the control. The consistent superior performance could be seen in all the measured traits except for the number of sylleptic shoots and crown length. In addition, genotype Bel37 had almost stable performance across the treatments. However, the phenotypic value of Bel37 was generally lower than the mean value. Even though genotype IÅ111 and genotype IÅ143 came from the same country – Sweden, they had different performance in the treatments. Also, for some genotypes (e.g. genotype 14, genotype 82 and genotype 103), they only showed significant difference across the treatments in one of the traits.

It was interesting to discover that some genotypes (e.g. genotype 111) are sharing the same genetic profile despite they are from different countries (e.g. Great Britain and Poland) (data provided by Johan Fogelqvist).

Table 4: ANOVA analysis based on Model 3. Variance components were used for the calculation of broad sense heritability (H^2) and coefficient of variance (CV_G) in control and drought.

Phenotypic traits	Control [1]				Drought [1]			
	σ^2_G	σ^2_ε	$H^2_{Genotype} \pm SE$	CV_G (%)	σ^2_G	σ^2_ε	$H^2_{Genotype} \pm SE$	CV_G (%)
Height growth during treatment	257.16	791.17	0.49 ± 0.03	13.83	133.93	1287.16	0.24 ± 0.04	11.99
Crown length	254.78	908.00	0.46 ± 0.04	13.29	45.86	1014.59	0.12 ± 0.04	6.98
Number of sylleptic shoots	7.39	36.97	0.37 ± 0.04	54.32	4.13	23.45	0.35 ± 0.04	65.58
Stem dry weight	5.53	13.88	0.54 ± 0.03	27.71	0.83	9.54	0.21 ± 0.04	16.24
Leaf dry weight	1.50	3.85	0.54 ± 0.03	24.34	0.19	3.22	0.15 ± 0.04	11.19
Total above-ground dry weight [2]	12.42	30.63	0.55 ± 0.03	26.00	1.42	22.56	0.16 ± 0.04	12.51
Stem biomass allocation (i.e. SBAI[3])	1.50×10^{-3}	3.19×10^{-3}	0.58 ± 0.03	6.53	1.61×10^{-3}	8.62×10^{-3}	0.36 ± 0.04	6.92

[1] σ^2_G – Genotype Variance; σ^2_ε – Residual Variance; H^2 – Broad sense heritability; **SE** – Standard error; CV_G – Coefficient of Variance

[2] **Total above ground dry weight** – Total of stem and leaf dry weights

[3] **SBAI** – Stem biomass allocation index

Overall, the broad sense heritability (H^2) decreased in the drought treatment **(Table 4).** In both treatments, the highest value of $H^2_{Genotype}$ was observed in stem biomass allocation. It was interesting to observe that $H^2_{Genotype}$ of the number of sylleptic shoots did not differ much in drought treatment; while the $H^2_{Genotype}$ of the other traits decreased drastically in drought treatment. Also, in drought treatment there was an increase in the residual variances (σ^2_ε) of height growth during treatment and crown length; while the other traits' residual variances experience reduction.

CV_G is an estimation of the standardized population genetic variance. All the traits experienced a decrease in CV_G values across the treatments except for the number of sylleptic shoots and stem biomass allocation. The number of sylleptic shoots had the highest CV_G values in both treatments (i.e. control = 54.32% and drought = 65.58%).

Table 5. Phenotypic correlation analysis in two treatments – control (blue) and drought (red). The strength of the correlation was shown in the r-value.

	HGDT	Crown length	NSS	Stem dry weight	Leaf dry weight	TARW	SBAI
[1]HGDT		r = 0.9275 ***	r = 0.0604 NS	r = 0.7962 ***	r = 0.7200 ***	r = 0.7723 ***	r = 0.3232 ***
Crown length	r = 0.9317 ***		r = 0.1019 NS	r = 0.7416 ***	r = 0.7610 ***	r = 0.7569 ***	r = 0.2379 ***
[2] NSS	r = 0.4022 ***	r = 0.3941 ***		r = 0.2551 ***	r = 0.1852 **	r = 0.2374 ***	r = 0.1824 **
Stem dry weight	r = 0.8318 ***	r = 0.7949 ***	r = 0.6021 ***		r = 0.8325 ***	r = 0.9623 ***	r = 0.5174 ***
Leaf dry weight	r = 0.8166 ***	r = 0.8284 ***	r = 0.5735 ***	r = 0.9372 ***		r = 0.9353 ***	r = 0.1187 *
[3] TARW	r = 0.8300 ***	r = 0.6130 ***	r = 0.5996 ***	r = 0.9889 ***	r = 0.9715 ***		r = 0.3904 ***
[4] SBAI	r = 0.7663 ***	r = 0.6660 ***	r = 0.4651 ***	r = 0.7500 ***	r = 0.6091 ***	r = 0.7141 ***	

[1)] **HGDT** - Height growth during treatment; [2)] **NSS** - Number of sylleptic shoots; [3)] **TARW** – Total above-ground dry weight (i.e. total of stem and leaf dry weights); [4)] **SBAI** - Stem biomass allocation index

NOTE: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS – Non significance

In the correlation analysis, all the traits showed positive correlation with each other in both treatments **(Table 5)**. However, the strength of correlations (*r*) was weaker in the drought treatment compared to the control. The correlation is only informative if there is a change in the gradient of the regression line across the treatment. All the correlation between the traits experience the slope gradient change, expect for the correlation between height growth during treatment and crown length, total dry weight and stem or leaf dry weight (data not shown).

The phenotypic correlation between total dry weight and stem dry weight was the strongest in both treatments. In well-watered condition, the number of sylleptic shoots had relatively weak but significant phenotypic correlation with growth traits (i.e. height growth during treatment and dry weight). In contrast, in drought treatment, there were no significant correlations of the

number of sylleptic shoots with height growth during treatment and crown length. In addition, stem biomass allocation had positive correlation with height growth during treatment, crown length and dry weight in both treatments.

5. Discussion

Overall, the results from this project suggest that there is phenotypic variation among *S. viminalis* genotypes in well-watered condition as well as in drought condition. The drought treatment imposed about 30% reduction of the total biomass production. Also, this study shows that different *S. viminalis* genotypes had different growth response in well-watered and drought conditions. Wikberg and Ögren (2004) suggested that within a population, the variation in drought tolerance in different genotypes differs due to the differences in water use efficiencies and transpiration rates in each genotype. This implies that certain genotypes could tolerate water stress better than the other. This suggests that within *S. viminalis* population, there were phenotypic variations in response to drought.

5.1 ***Height growth during treatment and crown length***

Crown length is the leafy part of the shoot. In this project, it served as the indirect indicator of leaf wilting. Leaf wilting is a kind of drought response, which is caused by irreparable xylem cavitation (Pockman and Sperry 2000). When water availability is limited, air bubbles enter root system due to pressure gradient and causes xylem to cavitate which leads to embolism (air filling). Repeated cavitations cause blockage in xylem and as a result, leaf wilts due to insufficient water supply (Wikberg 2006). If the shoot had a short crown length, it implies that there are many leaves wilting during the treatment. From our results, it showed that water shortage caused leaf wilting which was illustrated by the shorter crown length in drought treatment.

As shown in the correlation analysis, height growth during treatment and crown length had a very strong positive phenotypic correlation in both treatments. Also, both traits had the highest GxT quotient. This suggests that in these traits, there were more GxT interaction among *S.*

viminalis genotypes. In other words, there were more changing of the genotype ranking within *S. viminalis* population across the treatments.

In the heritability analysis, both traits had relatively high $H^2_{Genotype}$ in the control. This suggests that in well-watered condition, much of the phenotypic variation is attributed to the genetic factors, while the environmental factors have less influence on the phenotypic variation. The broad sense heritability value (H^2) for height growth in well-watered condition was higher than the one that was reported by Lin and Zsuffa (1992) in *S. eriocephala.* This is because the studied population is different and also the difference in H^2 estimation formula. Lin and Zsuffa (1992) included the site and block effect in the H^2 estimation which could eliminate overestimation of heritability.

Also, the heritability reduced in drought condition and crown length experienced greater reduction compared to the height growth during treatment. In addition, this suggests that the genetic factors that control crown length are more affected by drought compared to height growth. The decreased heritability in drought treatment suggests that during water stress condition, these traits are less influenced by genetic factor and thus, they are not highly heritable in drought. Furthermore, the genetic variation of these two traits decreased in drought treatment. This implies that during water stress condition, these two traits have less genetic variability in *S. viminalis* population.

5.2 ***Number of sylleptic shoots***

In general, the results suggest that the number of sylleptic shoots is affected by water stress condition. We observed that there was less number of sylleptic shoots in drought condition. This is probably because the majority of the growth resources were allocated to the roots for the improvement of water uptake efficiency (Weih *et al.* 2011). As a result, less growth resource was channeled to sylleptic shoots and caused reduction in the number of sylleptic shoots.

It was interesting to find out that despite the low heritability in both treatments, the $H^2_{Genotype}$ values did not fluctuate much between the treatments. This suggests that the genetic factors that control the number of sylleptic shoots have a stable gene expression across the treatments. Moreover, the CV_G value increased in the drought treatment. This implies that during drought condition, this trait has higher genetic variation within the population.

Populus and *Salix* belong to the same taxonomic family. Therefore, *Populus* could be used as a reference for the study of *Salix*. In *Populus*, syllepsis has positive effect on stem size and biomass (Ceulemans *et al*. 1990). Also, in another study of *Populus*, it showed significant phenotypic correlation between syllepsis and plant growth (Marron *et al.* 2006). In addition, Marron *et al.* (2006) also found that the most productive *Populus* genotypes had more sylleptic shoots compared to the less productive genotypes. In our study, the strength of phenotypic correlation between the number of sylleptic shoots and stem dry weight was moderate but significant in the well-watered condition. This suggests that syllepsis could affect stem growth which is important for the biomass production. William and Pearna (2006) suggested that sylleptic shoots could contribute to the overall photosynthesis area by increasing the canopy size. Also, Marks (1975) proposed that sylleptic shoots is a characteristic of early vigour which will lead to rapid growth at the later growing stage. Although the sylleptic shoots drop off at the end of growing season, we hypothesized that sylleptic shoots might positively influence the

growth of *S. viminalis* which is important for the biomass production. Thus, sylleptic shoots could be considered as the indirect indicator of biomass production in *Salix*. Further study on how syllepsis affects *Salix* biomass production is required.

5.3 ***Dry weight***

In this experiment, stem and leaf dry weights were measured and the total was considered as the above-ground biomass. Overall, the results suggest that water stress has negative impact on biomass. Weih *et al.* (2011) suggested that during water stress condition, most of the resources will be channeled to root to promote root growth for water uptake. Even though this re-allocation will increase drought tolerance, it reduced the allocation of resource to the stem and leaves. As a result, the harvestable biomass decreased. Thus, we believe that reduction in dry weight might be due to re-allocation of resources to roots. However, we could not verify this hypothesis since root dry weight was not measure in this study.

Stem, leaf and total dry weights had the highest $H^2_{genotype}$ in the control. However, the values decreased drastically in drought and were among the lowest as compared to the other traits. This suggests that in drought condition, the phenotypic variation of dry weight in the population is mostly attributed to the environmental factor. Thus, there is less genetic influence in the phenotypic variation. Also, in the GxT Quotient, the GxT variance component (σ^2_{GxT}) was much higher than the genetic variance component (σ^2_G). This suggests that there is more GxT interaction among the genotypes and this implies that dry weight is more sensitive to environmental change.

According to Mitra (2001), during water stress condition, shortage of water supply could restrict optimal gene expression of a crop. In other words, favorable condition promotes optimal gene expression. Since drought is an abiotic stress and is an unfavorable condition, the

genetic factors that control dry weight might not be able to have optimal gene expression. Since dry weight is the quantitative trait, meaning that there is more than one gene that controls the genotypic expression of the trait. Thus, during water stress condition, some of the genes that express the trait do not have optimal expression. As a result, during water stress condition the genetic variability (CV_G) within *S. viminalis* population decreased.

5.4 ***Stem biomass allocation***

Stem is the most valuable part of *S. viminalis* because it is the main source for bioenergy production. Thus, it would be interesting to study how much biomass that is allocated to stem change from well watered to water-stress condition. Stem biomass allocation (i.e. SBAI) did not have much change between the treatments. This suggests that water stress seems to have no significant effect on the allocation pattern in this studied population.

In addition, stem biomass allocation had the highest $H^2_{genotype}$ in both treatments. This indicates that the biomass allocation to stem is greatly dependent on genetic factors and is less affected to the environmental factors. Furthermore, GxT interaction was not significant in this trait. This suggests that stem biomass allocation is less sensitive to environmental change.

5.5 ***Country of origin***

In our study, all *S. viminalis* were from different European countries. Also, it was interesting to find out that despite several genotypes are from different countries, they share the same genotypic profile. For example, genotype 111 from Great Britain and Poland share the common genotypic profile even though they are from different countries. This suggested that there was an exchange of plant materials between countries since long time ago. Thus, they have the identical genetic profile.

5.6 ***Limitations***

One of the limitations was the small number of replicates for each genotype in each treatment. It would be better to have larger number of replicates since this would increase the reliability of the statistic tests where we analyzed differences between treatments for each genotype. Also, it was unavoidable that some technical errors happened during the data collection (e.g. missing data, data duplications). Moreover, in the stem and leaf drying process, some samples had mold growing due to mishandling. This might affect the dry weight measurement.

To improve a similar experiment, measurement of root dry weight to complement the stem and leaf dry weights as this could provide information on how biomass was allocated to root during drought condition. In addition, the growth response in well-watered and water-stress condition could be observed at different time points during the treatment. This could be a good indicator of how each genotype responds to well-watered and drought conditions in short and long time frames.

5.7 ***Breeding implications***

This study shows that the stem biomass allocation is highly heritable and less influenced by environmental change. This could be an advantage in breeding because the trait will have rapid response to selection and have a stable performance across a wide range of environments. Thus, we propose that stem biomass allocation should be considered in breeding. Also, we recommend that the number of sylleptic shoots should receive more attention in breeding because syllepsis is correlated to growth traits in well-watered conditions and have been shown in another study that it plays a crucial role in the growth of *Salix* (William and Pearna 2006). Moreover, the number of sylleptic shoots has high genetic variation which is important for breeding. This is because it can provide more genetic combinations for the trait improvement through breeding.

In breeding, understanding of the genetic structure of a trait is crucial. Quantitative traits such as height and weight are under polygenic control where each gene imposes certain level of additive effect to the phenotypic variation (Falconer 1989). Additive effect is one of the important factors for understanding the genetic gain in the selection. If the selected trait has high additive effect, the whole population will have faster response towards the selection. Rönnberg-Wästljung (2001) estimated the additive effect of height and weight by crossing *S. viminalis* from Sweden and Poland. It was found that height had high additive effect while weight has low additive effect. This suggests that if height is selected for breeding, the studied population will have faster response towards selection. However, since we did not estimate the additive effect in this study, we cannot draw the conclusion about this.

GxE interaction is one of the important factors in breeding. The change of genotype ranking across different environments will affect on how the breeders select the promising genotypes in different environments for higher yield. Lindgren (1984) recommended that if GxT quotient is more than 0.5, the interaction should be considered in breeding. In our study, it shows that the

quantitative traits - crown length, height growth and dry weight display higher GxT interaction effect across the treatments. This could be a challenge for the breeders as it is harder to estimate the yield. Thus, in order to have the optimal yield, breeders have to consider the zone breeding.

Also, our study shows that different genotypes have different drought responses. This suggests that each genotype might have different adaptation towards different level of drought severity. The performance (i.e. productivity) of each genotype could be examined at different time points during drought treatment. This could give an idea on how the genotypes respond to different level of drought severity. This would be useful for the targeted breeding in the diverse European environments. In this project, we identified several genotypes that were worth for further study. For example, genotype Bel37 had almost stable performance across the treatments. Even though it did not have the highest genotype ranking in both treatments, it had the consistent biomass production across the treatments. In order to fulfill the goal of breeding, it will be ideal if genotype Bel37 could produce high and stable yield in both conditions. Thus, genotype Bel37 could be considered for further field experiment studies. In addition, genotype IÅ111 showed superior performance in drought treatment. It will be interesting to conduct a genetic analysis on this genotype as this might lead to the finding of drought tolerance molecular markers for breeding.

The ability to recover from drought is important for the overall productivity of a crop. According to Savage and Cavender-Bares (2011), the ability to re-sprout after leaf shedding is considered one of the drought survival strategies of *Salix*. However, the study suggested that despite the studied *Salix* species had high drought tolerance, this did not indicate that the crops had also high recovery ability. In contrast, Malabuyoc *et al.* (1985) showed that there was a positive relationship between drought tolerance and recovery ability in the studied rice species. Since different *S. viminalis* genotypes might have different drought tolerance and drought recovery ability, we cannot conclude that the recommended genotypes (i.e. IÅ111 and Bel37) will

consistently produce high yield after exposure to water stress. Hence, in order to examine the drought recovery ability of *S. viminalis* genotypes that we studied, more thorough experiments should be performed.

6. Conclusion

Salix has received increased attention around the world and it is no longer the crop that only is used for baskets manufacture. Undoubtedly, *S. viminalis* is a promising bioenergy source in future. Base on our knowledge, this project is the first study of phenotypic variation in drought resistance in a large population of *S. viminalis*. We hope that this study is constructive to *Salix* breeding as the bioenergy crops.

Acknowledgment

'In an ocean, a sailor is searching for the brightest star in the sky. During cloudy days, whales, seabirds and rain drops were with him. He was grateful. After many dark nights, the sailor saw the bright round moon and a clear sky. He found. Finally, he saw the brightest star he was looking for…'

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